



Veterinary Microbiology

Development and validation of a modified TaqMan based real-time PCR assay targeting the *lipI32* gene for detection of pathogenic *Leptospira* in canine urine samples



Bruno Alonso Miotto^{a,*}, Aline Santana da Hora^b, Sueli Akemi Taniwaki^b, Paulo Eduardo Brandão^b, Marcos Bryan Heinemann^b, Mitika Kuribayashi Hagiwara^a

^a Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil

^b Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil

ARTICLE INFO

Article history:

Received 27 April 2017

Accepted 12 September 2017

Available online 2 December 2017

Associate Editor: Agnes Figueiredo

Keywords:

Leptospirosis

Dog

Urine

qPCR

lipI32

ABSTRACT

A modified TaqMan real-time polymerase chain reaction targeting a 138 bp fragment within the *lipI32* gene was developed to identify exclusively pathogenic *Leptospira* spp. in dog urine samples. Thirty-five samples from dogs with suspected clinical leptospirosis and 116 samples from apparently healthy dogs were tested for presence of leptospiral DNA using the TaqMan-based assay. The results were compared with those from a well-established conventional PCR targeting the 16S RNA encoding gene associated with nucleotide sequencing analysis. The overall agreement between the assays was 94.8% (confidence interval [CI] 95% 88–100%). The newly developed assay presented 91.6% (CI 95% 71.5–98.5%) relative sensitivity (22[+] *lipI32* PCR/24[+] 16S RNA and sequencing), 100% (CI 95% 96.3–100%) relative specificity and 98.7% accuracy (CI 95% 94.8–100%). The *lipI32* assay was able to detect and quantify at least 10 genome equivalents/reaction. DNA extracted from 17 pathogenic *Leptospira* spp., 8 intermediate/saprophytic strains and 21 different pathogenic microorganisms were also tested using the *lipI32* assay, resulting in amplification exclusively for pathogenic leptospiral strains. The results also demonstrated high intra and inter-assay reproducibility (coefficient of variation 1.50 and 1.12, respectively), thereby qualifying the newly developed assay as a highly sensitive, specific and reliable diagnostic tool for leptospiral infection in dogs using urine specimens.

© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author at: Laboratório de Microbiologia e Imunodiagnóstico, Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Rua Professor Orlando Marques Paiva 87, Butantã, zip code 05508-270, São Paulo, SP, Brazil.

E-mail: brunomiotto@hotmail.com (B.A. Miotto).

<https://doi.org/10.1016/j.bjm.2017.09.004>

1517-8382/© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Leptospirosis is a reemerging bacterial disease of global importance caused by pathogenic spirochetes of the genus *Leptospira*.¹ Pathogenic *Leptospira* is currently classified into 10 distinct genomospecies and more than 260 serovars,² which can affect a wide variety of mammalian species, including dogs and humans. Leptospiral transmission occurs through direct contact with contaminated biological material or indirectly through contact with contaminated environmental water resources.³ Thus, identification of infected animals is a key strategy for preventing the disease.

Canine leptospirosis is frequently reported worldwide^{1,4} and infected dogs manifest a broad spectrum of clinical symptoms, ranging from hepatic and renal failure, often accompanied by hemorrhagic and pulmonary disorders, to mild, self-limiting febrile illness.³ Although susceptible to many serovars, dogs can act as maintenance hosts for different pathogenic *Leptospira* spp., notably *L. interrogans* sv. *Canicola*, thus contributing to the spread of leptospires into the environment without overt clinical signs of infection.¹

The diagnostic approach for canine leptospirosis is predominantly based on serological evidence of infection, usually obtained through the microscopic agglutination test (MAT).⁵ However, use of MAT presents several limitations and the serological results should be interpreted with parsimony: MAT presents low sensitivity during the early stages of the disease, and multiple samples from both the acute and the convalescent phases are usually required for confirming the infection.^{6,7} Additionally, serum titers may therefore represent recent exposure to the pathogen instead of active infection,^{6,7} and the interpretation of serological data can be particularly confusing in relation to recently immunized dogs or those with unknown immunization status. The identification of chronically infected individuals using MAT can also be challenging, given that serum titers may not necessarily be associated with asymptomatic renal carriage of leptospires.³

Although culturing leptospires is still considered the eligible test to unmistakably confirm leptospiral infection, recovery of viable leptospires from culture media faces critical drawbacks, such as frequent contamination, fastidious growth of the pathogen and low diagnostic sensitivity,¹ which undermine its use in clinical and epidemiological studies. On the other hand, PCR has been successfully used to confirm leptospiral infection and enables early diagnosis of clinical leptospirosis,^{8,9} along with identification of dogs with asymptomatic urinary shedding of leptospires.^{10–13}

A great number of PCR assays targeting different protein-encoding genes have been developed for diagnosing human and animal leptospirosis.⁵ Different quantitative PCR protocols targeting the *lipl32* gene have previously been described^{11,14–16}; however, few studies have validated these assays for detecting leptospires in canine specimens.^{11,14} LipL32 is an abundant outer membrane protein that occurs exclusively in pathogenic *Leptospira* and presents highly pairwise DNA sequence identity among pathogenic species.¹⁷

The current report describes the development and validation of a modified quantitative PCR assay targeting the *lipl32* gene for identifying pathogenic *Leptospira* in canine urine samples.

Material and methods

Samples and isolates

Urine samples from 35 dogs with suspected clinical leptospirosis that were attended at the University of São Paulo Veterinary Hospital Service (Hovet FMVZ-USP) between 2013 and 2015 were used to evaluate the diagnostic sensitivity, specificity and accuracy of the TaqMan PCR assay. The dogs included in this study were considered suspected for acute leptospirosis when presenting high blood urea nitrogen (BUN) and creatinine levels (above 60 mg/dL and 1.4 g/dL, respectively), in association with two or more typical clinical manifestations of leptospirosis (hemorrhagic disorders, fever, jaundice, prostration or muscle weakness). An additional 116 urine samples from apparently healthy dogs that were being kept in two different local public shelters, which were collected during 2013, were also tested.

The Ethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (protocols 2706/2012 and 2406140614) approved all procedures involving animal manipulation in the current study.

The leptospiral strains used in this study were provided by the Bacterial Zoonosis Laboratory, Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo. The isolates (Table 1) were maintained in semisolid Fletcher medium (BD Diagnostics, Sparks, MD, USA) containing 1.5% agar (BD Diagnostics) at 28 °C under aerobic conditions, and were collected at mid-log phase.

Sample processing and DNA extraction

Urine samples were collected via urinary catheterization or cystocentesis and were centrifuged within 2 h after collection (6500 × g for 25 min at 25 °C) as previously described.^{16,18} The pellets were resuspended in 2 mL of sterile phosphate-buffered saline (PBS; 0.01 M PO₄; 0.1 M NaCl; pH 7.2) and all DNA extractions were processed not more than 48 h after collection. In order to access the performance of the DNA extraction procedures and verify the presence of PCR inhibitors, all clinical samples were subjected to quantitative amplification targeting the melanocortin 1 receptor-encoding gene (*MC1R*), a nuclear gene that encodes for a seven-pass transmembrane G protein-coupled receptor protein that is involved in hair and fur coloring in mammals.¹⁹ Genomic DNA from clinical samples was extracted using NucliSens[®] miniMAG[™] (BioMérieux Inc., Durham, NC, USA), in accordance with the manufacturer's instructions, with slight modifications: 1 mL of resuspended solution was used in the initial lysis step and the final elution step was performed with 40 µL.

Additionally, pure cultures of leptospires and non-spirochetal pathogens used to determine the analytical

Table 1 – Pathogenic, intermediate and nonpathogenic *Leptospira* strains used for sensitivity and specificity tests and results from TaqMan real-time PCR and 16S PCR assays.

Species	Serogroup	Serovar	Strain	TaqMan <i>lipL32</i>	PCR 16S
<i>Pathogenic</i>					
<i>L. interrogans</i>	Pomona	Pomona	pomona	+	+
	Canicola	Canicola	Hond Utrecht IV	+	+
	Icterohaemorrhagiae	Icterohaemorrhagiae	rga	+	+
	Icterohaemorrhagiae	Copenhageni	m20	+	+
<i>L. kirschneri</i>	Sejroe	Hardjo (Hardjoprajitno)	hardjoprajitno	+	+
	Autumnalis	Butembo	butembo	+	+
	Cynopteri	Cynopteri	3522c	+	+
<i>L. santarosai</i>	Pomona	Mozdok	m36/05	+	+
	Shermani	Shermani	1342k	+	+
	Grippothyphosa	Bananal	2a/cap	+	+
<i>L. borgpetersenii</i>	Sejroe	Guaricura	guaicururus	+	+
	Ballum	Castellonis	castellon 3	+	+
	Celledoni	Whitcombi	whitcomb	+	+
	Javanica	Javanica	veldrat batavia 46	+	+
<i>L. noguchii</i>	Mini	Mini	sari	+	+
	Panama	Panama	cz 214	+	+
	Panama		u73	+	+
<i>Intermediate/nonpathogenic</i>					
<i>L. inadai</i>	Lyme		m34/99	–	+
<i>L. meyeri</i>			19cap	–	+
<i>L. biflexa</i>	Doberdo	Rufino	rpe	–	+
	Seramanga	Patoc	patoc	–	+
	Nazaré	Nazaré	nazaré	–	+
	Andamana	Bovedo	bovedo	–	+
	Garcia	Buenos Aires	buenos aires	–	+
		Garcia	garcia	–	+

specificity of the *lipL32* TaqMan real-time PCR were subjected to two washing steps (6500 × g, 25 min at 25 °C) using sterile PBS prior to DNA extraction; the pellets were resuspended in 2 mL of sterile phosphate-buffered saline and genomic DNA was extracted using QIAamp DNA Mini Kit[®] (Qiagen Inc., Valencia, CA), in accordance with the manufacturer's instructions.

Primer and probe design

Primers and probe targeting the *lipL32* gene were selected based on a previously reported real-time PCR reaction described by Rojas et al.¹¹ A total of 113 sequences encoding the LipL32 protein that were available in the National Center for Biotechnology Information Nucleotide Database (NCBI; <http://www.ncbi.nlm.nih.gov/>) were aligned using the Mega 5.10 software²⁰ in order to evaluate the *in silico* specificity of the previously described primers. The selected sequences included all available *L. interrogans* sv. Canicola sequences, and sequences from *L. interrogans* serovars that are commonly attributed to canine leptospirosis (serovars Icterohaemorrhagiae, Pomona and Grippothyphosa), along with other representative serovars of the species *L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. noguchii*, *L. weilii* and *L. santarosai* (Supplementary Material 1). Two of the sequences thus selected (AY461924 and DQ286416) presented one nucleotide mismatch with the degenerated forward primer previously described.¹¹ Oligonucleotide structural analysis of the described primers was performed using OligoAnalyser 3.1 (<http://www.idtdna.com/>), which showed the possibility of self-dimer and hairpin formation. To accommodate

all available sequences tested and to overcome possible structural primer instability, a newly non-degenerated forward primer was designed (5'-TAAAGCCAGGACAAGCGCC-3'), resulting a 138 bp amplicon when combined with the reverse primer previously described, which was not modified. The fluorescent probe sequence described by Rojas et al.¹¹ was also not modified, except for use of minor groove binder (MGB) as a quencher at the 3'-end instead of nonfluorescent quencher (NFQ).

TaqMan real-time PCR assays

Optimal concentrations of primers (100, 300, 500, 600, 700 and 900 nM) and probe (200, 250 and 300 nM) targeting the *lipL32* gene were tested in accordance with the manufacturer's recommendations (Applied Biosystems, Thermo Fisher Scientific Inc., Carlsbad, CA, USA). This resulted in an optimized reaction with a final volume of 25 µL using 600 nM of each primer, 250 nM of the probe, 1× TaqMan[®] Universal Master Mix II (Thermo Fisher, Scientific Inc, Carlsbad, CA, USA), DNase-free water and 5 µL of extracted DNA. The amplification protocol consisted of 2 min at 50 °C, 10 min at 95 °C and 45 cycles of amplification (95 °C for 15 s and 60 °C for 60 s).

The MC1R assay was carried out using primers and probe previously described by Kanthaswamy et al.²¹ The reaction consisted of 1× TaqMan[®] Universal Master Mix II (Thermo Fisher, Scientific Inc, Carlsbad, CA, USA), 900 nM of each primer, 250 nM of the probe, sterile DNase-free water and 5 µL of extracted DNA, resulting in a final volume reaction

of 25 µL. The cycling conditions consisted of an initial step of 50 °C for 2 min, followed by 10 min at 95 °C and 40 cycles of amplification (95 °C for 15 s and 60 °C for 60 s). All MC1R runs were performed concomitantly with analysis on DNA extracted from pure cultures of canine fibroblasts, as positive controls.

All TaqMan real-time PCR runs were performed in the same machine (Applied Biosystems® 7500 Real-Time PCR System, Thermo Fisher Scientific Inc, Carlsbad, CA, USA).

Analytical sensitivity of the *lipI32* assay

The analytical sensitivity of the *lipI32* assay was determined through amplification of serial dilutions of genomic DNA extracted from a pure culture of *L. interrogans* serovar Canicola strain Hond Utrecht IV. DNA was extracted from a 2 mL sample and quantified in duplicate using a Qubit® 2 fluorimeter (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) with a Quant-iT™ DNA Assay Kit (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA). The number of Genomic Equivalents (GEs), assuming one genome copy per leptospire, was estimated based on a genomic size of 4.659 Mb. To prepare standard curves, the extracted DNA was standardized to an initial concentration of 1×10^6 GE/reaction, in order to perform serial 10-fold dilutions until reaching 1×10^1 GE/reaction. All standard-curve dilutions were tested in triplicate, and each run included a single negative control containing sterile nuclease-free water. The lower limit of detection (LLOD) was determined as the concentration at which 95% or more of the replicates from eight runs (performed on different occasions) presented amplification. The cycle threshold (C_t) cutoff value was determined by calculating the average C_t values of LLOD replicates from different standard-curve runs. Coefficients of variation (CV) and average C_t values were calculated to define inter and intra-assay variations and estimate the assay reliability. The CV values were calculated using the standard deviation (SD) and the mean C_t using the formula $CV = (SD \times 100\%) / \text{mean } C_t$.

Analytical specificity of the *lipI32* assay

The specificity of the new set of primers targeting the *lipI32* gene was initially confirmed by BLAST analysis (<http://blast.ncbi.nlm.nih.gov/>), followed by performing the assay using extracted DNA from several spirochetes as a template, including pathogenic strains, saprophytic strains and strains with intermediate pathogenicity. DNA extracted from pure cultures of other non-leptospiral pathogens was also tested, including *Brucella canis* (strain RM6/66 – ATCC 23365), *Enterococcus faecalis* (field strain), *Escherichia coli* (strain DH5α), *Salmonella enterica* sv. Typhi (ATCC 14028), *Yersinia enterocolitica* (field strain), *Proteus mirabilis* (field strain), *Candida albicans* (ATCC 90112), *Staphylococcus epidermidis* (field strain), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (field strain), *Klebsiella pneumoniae* (field strain), *Pseudomonas aeruginosa* (ATCC 27853), *Serratia marcescens* (field strain), *Mycobacterium tuberculosis* (strain H37Rv), *Mycobacterium bovis* (strain AN5), *Rickettsia rickettsii* (strain TAIACU), *Rickettsia bellii* (strain MOGI), *Rickettsia parkeri* (strain AT24), *Ehrlichia canis*

(strain SÃO PAULO), *Leishmania infantum chagasi* (strain CBT56 RIO GRANDE DO NORTE) and *Toxoplasma gondii* (strain RH).

Diagnostic sensitivity, specificity and accuracy of the *lipI32* assay

Quantitative amplification of DNA extracted from clinical samples that were obtained from symptomatic and asymptomatic dogs was performed in triplicate. The samples were considered positive when at least two thirds of the replicates presented C_t values lower than the cutoff value that had been established. For comparative analysis, the performance of the modified *lipI32* TaqMan real-time PCR assay was compared with results obtained from a well-established conventional PCR in which a *Leptospira* genus-specific set of primers targeting a 331 bp fragment of the 16S rRNA gene (Lep1 F and Lep2 R) was used.²² Conventional PCR amplification was carried out as follows: 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. Pure *L. interrogans* serovar Canicola (strain Hond Utrecht IV) genomic DNA was used as a positive control and DNase-free water as a negative control in all PCR runs. The amplified products were separated by electrophoresis on a 2% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) and were analyzed under UV transillumination. The nucleotide sequences of the amplified fragments were sequenced using the BigDye Terminator 3.1 cycle sequencing kit (Thermo Fisher Scientific Inc, Carlsbad, CA, USA) on an ABI 7500 Genetic Analyzer (Thermo Fisher Scientific Inc, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations. The sequences obtained were compared with those in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>), to determine species identification. Agreement between the assays was assessed using the kappa test, with a 95% confidence interval, along with calculation of relative sensitivity, specificity and accuracy. The 95% confidence intervals (CI) for the sensitivity and specificity values were calculated as proportion CI. The 95% confidence intervals for proportions (sensitivity, specificity and accuracy) were calculated, as all the other statistics, using the software RStudio (Version 1.0.136 – © 2009–2016 RStudio, Inc).

Results

The modified *lipI32* TaqMan real-time PCR assay was able to detect 1×10^6 to 1×10^1 GE/reaction of the extracted leptospiral DNA in a linear manner, with a coefficient of correlation (of 0.998 and efficiency of 98.96%. The analytical data also showed that the assay was able to detect 10 copies per reaction (LLOD): from 42 replicates containing 10 copies/reaction from eight different runs, 41 replicates presented amplification.

The reaction presented a C_t cutoff value of 37.5 arbitrary units (SD ± 0.324) and was determined based on the average C_t value of 17 replicates amplifying 10 copies per reaction from five different standard-curve runs. The standard curves were selected based on adequate efficiency values (ranging

from 92.74% to 98.96%) and high C_t values (> 38) were not considered in the calculation to avoid nonspecific amplifications. All selected runs were performed on separate days and the highest intra-assay and inter-assay CV values considering all standard-curve dilutions were 1.50 and 1.12, respectively (Supplementary Material 2).

The *lipI32* TaqMan real-time PCR assay was able to detect DNA from all pathogenic *Leptospira* spp. tested, while none of the intermediate or saprophytic *Leptospira* strains presented amplification (Table 1). All strains tested were also subjected to 16S rRNA amplification to confirm the appropriate extraction procedure, and all samples yielded strong positive bands when analyzed on agarose gel (Table 1). Moreover, the new set of primers used did not show any unspecific hybridization in the *in silico* evaluation and no amplification was observed when DNA from other non-spirochetal pathogens was tested.

When considering both symptomatic and asymptomatic dogs, the *lipI32* assay presented high overall sensitivity (91.6%; CI 71.5–98.5%), specificity (100%; CI 96.3–100%) and accuracy (98.7%; CI 94.8–100%), also revealing high agreement with the conventional PCR assay associated with sequencing analysis ($\kappa=0.948$; CI 0.88–1). All the parameters used to compare the *lipI32* TaqMan real-time PCR assay and the 16S PCR assay (with and without DNA sequencing), according to the clinical status of the dogs are presented in Table 2.

Out of the 35 urine samples collected from dogs presenting suspected acute leptospirosis, 11 (31.42%) showed positive results in the *lipI32* TaqMan real-time PCR assay. All *lipI32*-positive samples also tested positive in the 16S rRNA conventional PCR; DNA from all samples could be sequenced and BLAST analysis revealed high similarity ($>99\%$) to pathogenic *Leptospira* spp. However, five additional samples tested positive solely through the 16S rRNA amplification. Three of them presented high sequence similarity to non-leptospiral organisms (two in relation to uncultured bacteria and one in relation to *Canis lupus familiaris*), and two PCR-positive samples presented high sequence similarity to pathogenic *Leptospira* spp., thus revealing sensitivity differences between the *lipI32* TaqMan real-time PCR assay and the 16S assay performed with or without the association with sequencing analysis (Table 2). Among the 116 samples collected from apparently healthy dogs, 12 (10.34%) tested positive in the *lipI32* TaqMan real-time PCR assay; all *lipI32*-positive samples also tested positive in the conventional PCR and were successfully identified as pathogenic *Leptospira* species through further sequencing analysis ($>99\%$ similarity). Nevertheless, an additional 15 urine samples presented positive results solely through the 16S rRNA PCR (Table 2); for three samples, no readable sequences were retrieved, and sequence analyses on the other 12 samples revealed unspecific amplifications, with high sequence similarity to *Collinsella intestinalis* ($n=2$), *Corynebacterium* sp. ($n=2$), *Pasteurellaceae bacterium* ($n=1$), uncultured organisms ($n=6$) and *Canis lupus familiaris* ($n=1$).

All *lipI32*-negative samples tested positive for the MC1R gene, thus confirming that the DNA extraction and amplification procedures were appropriate.

Discussion

The modified qPCR assay described in this study presented high analytical sensitivity, thus enabling consistent detection of low concentrations of leptospiral DNA extracted from canine urine specimens (at least 10 copies per reaction). These results were compatible with the sensitivity levels of previous studies that used the *lipI32* gene as a target for molecular detection of leptospires in clinical samples.^{11,14,16} The reaction presented low analytical variation within or between runs, thus confirming the high analytical reliability of the assay.

The newly designed forward primer showed consensus areas in relation to all sequences retrieved from GenBank, including sequences from the two leptospiral strains belonging to *L. santarosai* and *L. borgpetersenii* that had presented nucleotide mismatches with the forward primer originally described.¹¹ Although unusual, *L. santarosai* and *L. borgpetersenii* infections were recently reported in dogs.^{23,24} Consequently, development of highly sensitive assays capable of detecting a more diverse range of pathogenic *Leptospira* sp. could be crucial for wider application of molecular assays. The *lipI32* TaqMan real-time PCR assay was able to detect DNA from all the pathogenic *Leptospira* spp. tested, while none of the intermediate and saprophytic strains presented amplification. Moreover, no *in silico* unspecific primer hybridization was detected and no amplification was observed when DNA from non-leptospiral pathogens was tested, thus confirming the high specificity of the assay.

All the positive TaqMan real-time PCR results obtained from clinical suspected cases were confirmed through the conventional 16S PCR when combined with further sequencing analysis. Despite the high overall agreement between the assays ($\kappa=0.874$), two samples tested positive solely through the 16S PCR. The nucleotide sequences obtained from both of these samples presented high similarity to the species *L. interrogans*, thus confirming leptospiral infection. The performance of the TaqMan real-time PCR assay was tested several months after running the 16S PCR, and therefore the discrepancy between these results might be explained by possible loss of DNA integrity during sample storage, as previously described.²⁵ Moreover, conventional PCR protocols are more susceptible to contamination during laboratorial procedures than are real-time assays,²⁶ and the differences observed might also be attributable to contamination while handling the clinical material. In relation to the *lipI32* TaqMan real-time PCR assay alone, 24 of the 35 dogs (68.6%) with suspected clinical leptospirosis did not present amplification of leptospiral DNA in urine samples. These results can be attributed to several causes, including: (I) intermittent urinary shedding of leptospires via urine; (II) hyperacute manifestation of the disease, thus preceding migration of the pathogen to the renal tubules; (III) clinical manifestations attributed to other causes not related to leptospiral infection; and (IV) shedding of very low quantities of leptospires in the urine, which would limit detection of the pathogen through the assay.

The molecular evaluation on urine samples taken from asymptomatic dogs showed 100% agreement between TaqMan real-time PCR and conventional PCR in association with nucleotide sequence analysis ($\kappa=1$). Nonetheless,

Table 2 – Kappa test and accuracy calculation and relative sensitivity/specificity values of the *lipI32* TaqMan real-time PCR assay using the 16S PCR assay and 16S PCR assay associated with *Leptospira* identification by DNA sequencing as reference tests.

Clinical status of the dogs			16S PCR assay			16S PCR + <i>Leptospira</i> sp. identification by sequencing		
			Negative	Positive	Total	Negative	Positive	Total
Symptomatic	TaqMan real-time assay	Negative	19	5	24	22	2	24
		Positive	0	11	11	0	11	11
		Total	19	16	35	22	13	35
			Sp = 100% (79.1–100%) Se = 68.8% (41.5–87.9%) kappa = 0.705 (0.48–0.93); accuracy = 85.7% (68.9–94.6%)			Sp = 100% (81.5–100%) Se = 84.6% (53.7–97.3%) kappa = 0.874 (0.70–1); accuracy = 94.3% (79.5–99.0%)		
Asymptomatic	TaqMan real-time assay	Negative	89	15	104	104	0	104
		Positive	0	12	12	0	12	12
		Total	89	27	116	104	12	116
			Sp = 100% (94.8–100%) Se = 44.4% (26.0–64.4%) kappa = 0.551 (0.36–0.74); accuracy = 87.1% (79.2–92.3%)			Sp = 100% (95.6–100%) Se = 100% (69.9–100%) kappa = 1.0 (1.0–1.0); accuracy = 100% (96.0–100%)		
Total	TaqMan real-time assay	Negative	108	20	128	127	2	129
		Positive	0	23	23	0	22	22
		Total	108	43	151	127	24	151
			Sp = 100% (95.7–100%) Se = 53.4% (37.8–68.5%) kappa = 0.593 (0.48–0.76) accuracy = 86.7% (80.0–91.5%)			Sp = 100% (96.3–100%) Se = 91.6% (71.5–98.5%) kappa = 0.948 (0.88–1.0) accuracy = 98.7% (94.8–100)		

Sp: relative specificity; Se: relative sensitivity.

15 samples tested positive solely through the conventional PCR assay, and sequencing analysis provided evidence of unspecific amplification in most of these samples ($n=12$). Despite several attempts, it was not possible to recover readable sequences or reproduce amplification from the other three samples, which were consequently considered negative. 16S ribosomal RNA gene sequencing is the most usual approach for molecular characterization of spirochetes and it is considered to be a well-established PCR-based tool for distinguishing leptospiral species.²⁷ However, previous reports have shown that there are considerable risks of amplifying DNA from certain commensal and environmental bacteria,¹⁴ which undermines its use for confirmation of leptospiral infection in clinical samples. Although the 16S PCR was shown to be remarkably unspecific in the present study, the results also showed that use of primers targeting the 16S rRNA gene can provide reliable results if associated with sequencing methods. Furthermore, all unspecific amplifications in 16S PCR tested negative in the quantitative PCR, which confirmed the high specificity of the newly developed qPCR assay.

The application of the TaqMan real-time assay using combined samples from both symptomatic and asymptomatic dogs also revealed high sensitivity, specificity and accuracy standards, with high overall agreement between the newly developed TaqMan assay and the conventional PCR associated with sequencing methods, as our results have shown.

In conclusion, the present study described the development and validation of a reliable and highly sensitive

quantitative assay targeting exclusively pathogenic *Leptospira* species. These results may contribute to a more precise diagnostic approach for identification of symptomatic and asymptomatic dogs infected by pathogenic leptospires.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We would like to thank Marcia Regina Franzolin of the Butantan Institute for providing bacterial strains. This work was supported by the Research Support Foundation of the State of São Paulo (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP; no. 2012/14681-7); B.A.M. holds a FAPESP grant (no. 2012/13022-0) and a PhD fellowship (no. 164284/2014) from the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjm.2017.09.004](https://doi.org/10.1016/j.bjm.2017.09.004).

REFERENCES

- Schuller S, Francey T, Hartmann K, et al. European consensus statement on leptospirosis in dogs and cats. *J Small Anim Pract.* 2015;56:159–179.
- Bourhy P, Collet L, Brisse S, Picardeau M. *Leptospira mayottensis* sp. nov., a pathogenic species of the genus *Leptospira* isolated from humans. *Int J Syst Evol Microbiol.* 2014;64:4061–4067.
- Andre-Fontaine G. Canine leptospirosis – do we have a problem? *Vet Microbiol.* 2006;117:19–24.
- Azocar-Aedo L, Monti G. Meta-analyses of factors associated with leptospirosis in domestic dogs. *Zoonoses Public Health.* 2016;63:328–336.
- Picardeau M, Bertherat E, Jancloes M, Skouloudis AN, Durski K, Hartskeerl RA. Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. *Diag Microbiol Infect Dis.* 2014;78:1–8.
- Miller MD, Annis K, Lappin MR, Lunn KF. Variability in results of the microscopic agglutination test in dogs with clinical leptospirosis and dogs vaccinated against leptospirosis. *J Vet Int Med.* 2011;25:426–432.
- Levett PN. Leptospirosis. *Clin Microbiol Rev.* 2001;14:296–326.
- Harkin KR, Roshto YM, Sullivan JT. Clinical application of a polymerase chain reaction assay for diagnosis of leptospirosis in dogs. *J Am Vet Med Assoc.* 2003;222:1224–1229.
- Harkin KR, Roshto YM, Sullivan JT, Purvis TJ, Chengappa MM. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. *J Am Vet Med Assoc.* 2003;222:1230–1233.
- Gay N, Soupé-Gilbert ME, Goarant C. Though not reservoirs, dogs might transmit *Leptospira* in New Caledonia. *Int J Environ Res Public Health.* 2014;11:4316–4325.
- Rojas P, Monahan AM, Schuller S, Miller IS, Markey BK, Nally JE. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *Eur J Clin Microbiol Infect Dis.* 2010;29:1305–1309.
- Samir A, Soliman R, El-Hariri M, Abdel-Moein K, Hatem ME. Leptospirosis in animals and human contacts in Egypt: broad range surveillance. *Rev Soc Bras Med Trop.* 2015;48:272–277.
- Zakeri S, Khorami N, Ganji ZF, et al. *Leptospira wolffii*, a potential new pathogenic *Leptospira* species detected in human, sheep and dog. *Infect Gen Evol.* 2010;10:273–277.
- Fink JM, Moore GE, Landau R, Vemulapalli R. Evaluation of three 5' exonuclease-based real-time polymerase chain reaction assays for detection of pathogenic *Leptospira* species in canine urine. *J Vet Diagn Invest.* 2015;27:159–166.
- Villumsen S, Pedersen R, Borre MB, Ahrens P, Jensen JS, Kroghfelt KA. Novel TaqMan® PCR for detection of *Leptospira* species in urine and blood: pit-falls of in silico validation. *J Microbiol Methods.* 2012;91:184–190.
- Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the *LipL32* gene. *Diag Microbiol Infect Dis.* 2009;64:247–255.
- Pinne M, Haake DA. *LipL32* is a subsurface lipoprotein of *Leptospira interrogans*: presentation of new data and reevaluation of previous studies. *PLOS ONE.* 2013;8:e51025.
- Levett PN, Morey RE, Galloway RL, Turner DE, Steigerwalt AG, Mayer LW. Detection of pathogenic leptospires by real-time quantitative PCR. *J Med Microbiol.* 2005;54:45–49.
- Evans JJ, Wictum EJ, Penedo MCT, Kanthaswamy S. Real-time polymerase chain reaction quantification of canine DNA. *J Forensic Sci.* 2007;52:93–96.
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol.* 2013;30:2725–2729.
- Kanthaswamy S, Premasuthan A. Quadriplex real-time PCR (qPCR) assay for human–canine–feline species identification and nuclear DNA quantification. *Forensic Sci Int Gen.* 2012;6:e97–e98.
- Mérien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J Clin Microbiol.* 1992;30:2219–2224.
- Miotto BA, Moreno LZ, Guilloux AG, et al. Molecular and serological characterization of the first *Leptospira santarosai* strain isolated from a dog. *Acta Trop.* 2016;162:1–4.
- Llewellyn JR, Krupka-Dyachenko I, Rettinger AL, et al. Urinary shedding of leptospires and presence of *Leptospira* antibodies in healthy dogs from Upper Bavaria. *Berl Munch Tierarztl Wochenschr.* 2016;129:251–257.
- Carlsen KH, Jensen JS. *Mycoplasma genitalium* PCR: does freezing of specimens affect sensitivity? *J Clin Microbiol.* 2010;48:3624–3627.
- Mackay IM. Real-time PCR in the microbiology laboratory. *Clin Microbiol Infect.* 2004;10:190–212.
- Postic D, Riquelme-Sertour N, Merien F, Perolat P, Baranton G. Interest of partial 16S rDNA gene sequences to resolve heterogeneities between *Leptospira* collections: application to *L. meyeri*. *Res Microbiol.* 2000;151:333–341.